Atmospheric Environment

Current ozone levels threaten gross primary production and yield of Mediterranean annual pastures and nitrogen modulates the response

Héctor Calvete-Sogo , Susana Elvira , Javier Sanz , Ignacio González-Fernández , Héctor García-Gómez , Laura Sánchez-Martín , Rocío Alonso , Victoria Bermejo-Bermejo

A B S T R A C T

Pastures are among the most important ecosystems in Europe considering their biodiversity and distribution area. However, their response to increasing tropospheric ozone (O_3) and nitrogen (N) deposition, two of the main drivers of global change, is still uncertain. A new Open-Top Chamber (OTC) experiment was performed in central Spain, aiming to study annual pasture response to O_3 and N in close to natural growing conditions. A mixture of six species of three representative families was sowed in the field. Plants were exposed for 40 days to four O_3 treatments: filtered air, non-filtered air (NFA) reproducing ambient levels and NFA supplemented with 20 and 40 nl l^{-1} O_3. Three N treatments were considered to reach the N integrated doses of "background", +20 or +40 kg N ha^{-1}. Ozone significantly reduced green and total aboveground biomass (maximum reduction 25%) and increased the senescent biomass (maximum increase 40%). Accordingly, O_3 decreased community Gross Primary Production due to both a global reduction of ecosystem CO_2 exchange and an increase of ecosystem respiration. Nitrogen could partially counterbalance O_3 effects on aboveground biomass when the levels of O_3 were moderate, but at the same time O_3 exposure reduced the fertilization effect of higher N availability. Therefore, O_3 must be considered as a stress factor for annual pastures in the Mediterranean areas.

1. Introduction

Tropospheric ozone (O_3) and atmospheric nitrogen (N) deposition are two of the main air pollutants affecting natural and semi-natural areas and causing harmful ecological effects (EEA, 2011; Sutton et al., 2011). In the Mediterranean area, high solar radiation, temperature and prevailing stable atmospheric conditions favor photochemical O_3 formation (Millán et al., 2000; Cristofanelli and Bonasoni, 2009), resulting in some of the highest surface O_3 concentrations in Europe (EEA, 2011). Ozone concentrations in Spain frequently exceed current thresholds established for plant protection according to the EU Air Quality Directive 2008/50/EC or the Convention on Long-Range Transboundary Air Pollution of the UN/ECE (CLRTAP) (Fernández-Fernández et al., 2011). Moreover, O_3-induced effects have been reported in crops and natural

* Corresponding author. Ecotoxicology of Air Pollution, CIEMAT (Edif. 70), Avda. Complutense 40, 28040 Madrid, Spain.

E-mail addresses: hector.calvete@ciemat.es (H. Calvete-Sogo), susana.elvira@ciemat.es (S. Elvira), j.sanz@ciemat.es (J. Sanz), ignacio.gonzalez@ciemat.es (I. González-Fernández), hector.garcia@ciemat.es (H. García-Gómez), laura.sanchez@upm.es (L. Sánchez-Martín), rocio.alonso@ciemat.es (R. Alonso), victoria.bermejo@ciemat.es (V. Bermejo-Bermejo).

vegetation (Ferretti et al., 2007; Bermejo et al., 2011). Total atmospheric N deposition in Spain reaches values up to 23–30 kg N ha^{-1} $year^{-1}$ (Àvila and Rodà, 2012; García-Gómez et al., 2014). These values are a relatively lower than deposition levels recorded in central Europe. However, since changes in species composition occur early in the sequence of N saturation (Emmett, 2007), N deposition effects could be occurring in Spanish natural ecosystems. Unfortunately, despite the presence of extraordinary biological richness in the Mediterranean Basin, very little information is available on the threat that air pollution and in particular reactive N, can pose to biodiversity in this area (Ochoa-Hueso et al., 2011).

Most of the research on ecosystems responses to air pollution has been focused on the effects of a single pollutant while air pollutants seldom occur individually. Interactive effects of O_3 and N deposition are still poorly understood. Higher N availability can increase plant productivity providing higher resource availability for investing in defense, repair and compensation processes counterbalancing O_3 effects, or may exacerbate O_3 damage due to stimulated growth and higher specific leaf area (Bytnerowicz, 2002; Ashmore, 2005; Bassin et al., 2007). On the other hand, O_3 can inhibit some N-related enzyme activities and reduce the allocation of assimilates to roots and root growth (Bytnerowicz, 2002; Ashmore, 2005), thus diminishing the fertilization effects of higher N levels.

Grasslands communities represent an important contribution to global biodiversity. Experiments with single species have shown a wide range of O_3 sensitivity (Bassin et al., 2007; Hayes et al., 2007). However, the response of plant communities cannot readily be predicted from the response of their component species, and little information is available on the effects of O_3 on pasture communities (Bassin et al., 2007; Cape, 2008). Communities of temperate perennial grasslands have shown higher resilience to O_3 compared to individual species (Thwaites et al., 2006; Volk et al., 2011). On the other hand, chronic N deposition is reducing plant species richness of European temperate acid grasslands (Stevens et al., 2004; Dupre et al., 2010). The combination of O_3 and N deposition on perennial grasslands shows contrasting results with no interactive effects or an additive response in C allocation in some species (Volk et al., 2011; Wyness et al., 2011).

Fewer works have been focused on the response of annual pastures to air pollution. Annual grasslands constitute the understorey of broadleaf evergreen forests, and dehesa traditional agroforestry systems. These ecosystems are protected by the 92/43/EEC Habitat Directive and included in the Nature 2000 network. Previous experiments with individual species grown in pots showed that O_3 induces visible injury, accelerates senescence and reduces biomass production and consumable food value of some representative species (Bermejo et al., 2003; Gimeno et al., 2004a,b; Sanz et al., 2005, 2007, 2011). Interestingly, nitrogen fixing legumes, with high nutritive quality for herbivorous feeding, were more O_3 sensitive than grasses (Bermejo et al., 2003; Gimeno et al., 2004a). Moreover, O_3 strongly reduced flower and seed production of some sensitive legumes (Gimeno et al., 2004b; Sanz et al., 2007) which could interfere with species perpetuation and competitive fitness. For some species, N fertilization counterbalanced O_3 effects on senescence and plant and flower biomass, when plants were exposed to moderate O_3 concentrations (Sanz et al., 2005, 2007, 2011). Experiments with annual plant communities are needed to test if results obtained with individual species grown in pots are applicable for O_3 risk assessment to annual grassland communities.

An experiment has been carried out to study the interactive effects of O_3 and N fertilization on a simplified annual community. The main hypotheses were that O_3 affects community gas exchange rates reducing biomass production, and that N can modulate the negative response to this pollutant. To our knowledge this is the first experiment providing results of effects of O_3 and N fertilization on an annual grassland community.

2. Materials and methods

2.1. Experimental site

The experiment was performed in a new Open Top Chamber (OTC) facility located in the Spanish central plateau at Santa Olalla (450 m.a.s.l.; 40°3′N, 4°26′W), in the scientific experimental farm "La Higueruela" (CSIC). The area presents a Thermo-Mediterranean temperate climate, with moderate winters and dry summers. The field site is far from local sources of air pollution and at 80 km downwind of Madrid City. The soil is a calcic luvisol, characterized by differentiation pedogenetic of clay (11.4% clay, 24% silt, 64.6% sand). Further information of edaphic and physic-chemical characteristics of Ap horizon is provided in Appendix (Table A.1.)

2.2. Plant material

A simplified pasture community was sown on the ground using 6 representative annual species: 3 legumes (*Trifolium striatum*, *Trifolium cherleri*, *Ornithopus compressus*), 2 grasses (*Briza maxima*, *Cynosurus echinatus*) and 1 forb (*Silene gallica*) (Table 1). The family composition followed a proportion of 45% leguminous, 45% grasses and 10% caryophylaceae. Legumes and grasses are the most abundant species in natural annual pastures (Montoya et al., 1988). Species selection was based on natural abundance, relatively homogeneous seed size to allow homogeneous sowing, seed availability, high seed germinative capacity and O_3-sensitivity based on previous studies (Bermejo et al., 2003; Gimeno et al., 2004; Sanz et al., 2007). Before the experiment, in order to reduce plants growing from the natural soil seed bank inside the experimental plots, seeds present in the soil were successively allowed to germinate and seedlings were eliminated afterwards turning over 20 cm of top soil. Experimental seeds were sown on February 11th 2011, aiming for a density of 1000 plant m^{-2} using the proportion of species seeds indicated in Table 1; the sowing mixture was adjusted according to seed weight and germination capacity tested previously. The homogeneity of species germination and distribution inside the chambers was tested and confirmed 5 days after emergence (DaE). Every week, alien species were manually removed.

2.3. Experimental design

The experiment followed a split-plot design conducted in 15 plots with four O_3 treatments three times replicated (i.e. a total of 12 OTCs with 3 OTCs per O_3 treatment) and three ambient plots without chamber. Four O_3 treatments were considered: charcoal filtered air (FA), non-filtered air (NFA) reproducing ambient levels, non-filtered air supplemented with 20 nl l^{-1} O_3 (NFA+) and non-filtered air supplemented with 40 nl l^{-1} O_3 (NFA++). Ambient air chamberless plots (AA) were considered to evaluate the chamber effect. The experimental unit was the NCLAN-type OTC (adapted from the original OTC design used in the National Crop Loss Assessment Program of the Environmental Protection Agency, USA; Heck et al., 1982). Ozone supply for NFA+ and NFA++ treatments was applied during 8 h day^{-1} (7:00 to 15:00 GTM) 7 day $week^{-1}$ by mean of an O_3 generator (Model 16, A2Z Ozone Systems Inc., USA) system fed with pure oxygen. Ozone (ML® 9810B, Teledyne, USA), sulfur dioxide (SO_2; ML®9850B UV, Teledyne, USA), and nitrogen oxides (NO_2 and NO; ML®9841, Teledyne, USA) concentration inside each chambers and AA plots were monitored continuously above

Table 1
Species composition of the experimental annual pasture community.

Species	Family	Seed source	Percentage in the mix[c]	Seed germinative rate (%)	100 Seed weight (g)	Ozone sensitivity	Reference
Trifolium striatum	*Leguminosae*	Seed bank[a]	15	88	0.234	sensitive	Sanz et al., 2007
Trifolium cherleri	*Leguminosae*	Seed bank[a]	15	96	0.285	sensitive	Sanz et al., 2007
Ornithopus compressus	*Leguminosae*	Seed bank[a]	15	57	0.156	resistant	Bermejo et al., 2003
Cynosurus echinatus	*Gramineae*	D. Moncalvillo[b]	22.5	88	0.133	resistant	Gimeno et al., 2004a,b
Briza maxima	*Gramineae*	D. Moncalvillo[b]	22.5	93	0.273	Rel. resistant	Sanz et al., 2011
Silene gallica	*caryophylaceae*	D. Moncalvillo[b]	10	76	0.041	unknown	

[a] Bank of Extremadura Community Agriculture Department (seeds from central-western areas of the Iberian Peninsula; *T. striatum*: 38°55′N, 05°06′W; *T. cherleri*: 38°22′N, 0°64′W; *O. compressus*: 38°55′N, 05°06′W).
[b] Dehesa de Moncalvillo, Guadalix de la Sierra, Madrid (40°40′N, 03°46′W).
[c] Weight based.

the canopy (50 cm above the soil) using an automated time-sharing system which sampled each plot for 10 min. The accumulated O_3 exposure was characterized by the AOT40 index representing the accumulated exposure over a threshold of 40 nl l^{-1} during daylight hours. Micro-meteorological stations were installed inside the AA plots, FA and NFA++ chambers for continuously monitoring air relative humidity (RH) and temperature (HOBO® Pro v2, Onset), photosynthetic active radiation (PAR; OSO-SUN HOBO®, Onset, USA), soil relative humidity (ECHO 10, Decagon Devices, USA) and soil temperature (TMC6-HD HOBO®, Onset, USA) at 10–15 cm depth.

Immediately after seed sowing (February 11, 2011), all experimental plots were divided in three sectors (1.4 m^2 each) delimiting the three N input treatments: N0 (soil N background), N20 (20 kg N ha^{-1}) and N40 (40 kg N ha^{-1}). To reach these integrated doses, N supplementation was applied every 2-weeks using an ammonium nitrate (NH_4NO_3) solution (Table 2). Trying to keep the natural limited water conditions of annual pastures, watering was only lightly applied when strictly necessary, i.e. after sowing, with N applications (3 l water m^{-2} for each N application) or when an excessive water stress was detected that could compromise the experiment. Nitrogen content of the water supplied was periodically controlled being always close to zero. A total volume of 48 l water m^{-2} during the whole experimental period was applied; thus, incrementing about 30% the natural precipitation experienced during the growing season.

Table 2
Timetable of the experimental events.

Event	Date	DaE[a]	DaS[b]
Sowing	11-Feb		
Emergence	25-Feb	1	
First harvest (vegetative stage)	11-Apr	46	0
Start of ozone exposure	12-Apr	47	1
Gas exchange sampling (vegetative stage)	12/14 Apr	47 (46–48)	0–2
First N fertilization	13-Apr	48	2
Second N fertilization	26-Apr	61	15
Gas exchange sampling (reproductive stage)	27/28 April	62 (62–63)	16–17
Third N fertilization	10-May	75	34
Second harvest (reproductive stage)/leaf damage quantification	20-May	85	39
Fourth N fertilization	24-May	89	43
Gas exchange sampling (senescence stage)	25–27 May	91 (89–93)	44–48
End of ozone exposure	30-May	95	49
Third harvest (senescence and seed maturity)	20-Jun	116	70

Numbers in brackets indicate the real range of the sampling period for a particular parameter.
[a] DaE = Days after emergence.
[b] DaS = Days after start of ozone exposure.

Plant exposure to the different O_3 treatments started in April, 47 days after emergence (DaE; Table 2). Almost at the same time (48 DaE), the first N fertilization was applied. Pasture was exposed to O_3 for 49 days until the community reached its maximum development and productivity, then the O_3 exposure system was switched off allowing plants to dry up and complete seed maturation which happened at the end of May.

Total soil mineral N (N_{min}) content ($NO_3^- + NH_4^+$) was analyzed throughout the experiment within the top 20 cm of the upper soil horizon. After the extraction of 8 g of mixed fresh soil with 50 ml of water and 50 ml of 1 M KCl solution, NO_3^- and NH_4^+ concentrations were measured with an Orion 720A electrode (Thermo Fisher Scientific, Beverly, MA, USA) and with a Technicon AAII Auto-analyser (Technicon Hispania, Madrid, Spain) respectively.

2.4. Visible injury

Visible leaf damage was evaluated on the second harvest (Table 2) based on the percentage of damaged leaves per plant. All the plants inside one sampling harvest ring (5 dm^2) per chamber and N treatment were evaluated. Ozone-induced foliar damage was quantified as the percentage of damaged leaves per plant relative to the total considering four classes: no injury, 0–25%, 25–50%, 50–75% and 75–100% of leaves affected. A leaf damage index for the community (LDI_c) was calculated based on the foliar mean damage for each individual species weighted by its own abundance. Abundance per species was estimated from their green biomass proportion inside the sampling ring.

2.5. Biomass harvest

Aboveground biomass production was harvested three times throughout the life cycle of the pasture (Table 2). The early harvest was done just before the start of O_3 treatments and N applications (46 DaE), signaling the initial stage of the pasture (Vegetative Stage). The second harvest was collected after 39 days of O_3 exposure (85 DaE), when the pasture reached its maximum biomass and flowering development (Reproductive Stage). After this harvest, the O_3 fumigation system was switched off coinciding with the start of pasture natural decline. The last harvest was done when plant biomass was completely dried and seeds reached maturity (116 DaE, Senescence and seed maturity Stage). For each harvest, all plants within a 5 dm^2 sampling ring were cautiously collected per chamber and N treatment. Plant material was classified into species (data not shown) and immediately weighed to obtain fresh weight biomass. Afterwards, samples were dried to constant weight at 60 °C. Additionally, in the second harvest, green and senescent leaves were separated and root biomass was extracted by hand from the first 10 cm of soil collected inside the sampling ring.

2.6. Gas exchange at canopy level

Gas exchange at canopy level was measured using a steady-state custom-made cuvette coupled to the Li-Cor 6400 portable gas exchange system (Li-Cor, Nebraska, USA) following the company technical recommendations (García et al., 1990). A cylindrical cuvette was made of transparent methacrylate (24.5 cm radius, 29.4 cm height), covered with a polypropylene film to moderate temperature increments. The inside walls of the cuvette were coated with Teflon® tape to avoid condensation and water absorption and desorption on the walls. The open bottom allows the cuvette to be placed over the pasture. Two fans were set inside to facilitate air mixing. PAR (LI-190, LI-COR Inc, USA), air temperature and RH (Vaisala Inc. USA) were continuously monitored inside the cuvette. Air flow to the cuvette was propelled using an external pump (KNF NMP 830 KVDE 12V, KNF) and the inlet air flow was continuously monitored with an air velocity transducer (TSI 8455, TSI Inc., USA).

Gas exchange measurements were performed between 9:00–11:00 h GMT on clear sunny days at the three different pasture development stages (Table 2). During the Reproductive Stage, NFA was not measured due to bad weather conditions. Gas exchange measures were acquired only when the steady-stationary state was achieved which took around 2 min. Afterwards, instantaneous values were averaged for 2 min. Bare soil measures without plants inside the sampling ring were done at Senescence Stage in plots previously harvested and left without plants for one month. Ecosystem CO_2 Exchange (NEE) was calculated on ground surface area basis (μmol CO_2 m^{-2} s^{-1}) and on dry biomass basis (μmol CO_2 g dw^{-1} s^{-1}) using the biomass harvest data collected at the different phenological stages. After each full-light steady-state NEE measurement, cuvette was completely darkened with a black cloth to record the steady-state ecosystem Dark Respiration (R_{eco}). Gross Primary Production (GPP) was estimated according to: GPP = NEE−R_{eco}. Two measures per OTC and N treatment were done. After the measurements, plants inside the cuvette were harvested for biomass analyses.

2.7. Statistical analysis

The effects of N fertilization and O_3 exposure on the different response parameters and harvests were evaluated with Analysis of Variance (ANOVA) using a split-plot design, considering O_3 as the main factor, N as sub-factor and their interaction. A block effect was also included as a random factor. A phenological stage factor was considered to analyze gas exchange rates. Annual pastures did not grow again after harvest, thus different sampling rings were selected for each harvest assuring its independence for statistical purposes. When significant differences among treatments were detected ($p < 0.05$), mean differences were assessed with the Tukey Honestly Significant Difference test (HSD). Normal probability plots and scatter plots of residuals were used to test normality. The Levenne test was applied to check variance homoscedasticity. When data did not fulfill model requirements, data were log-transformed. Outliers were rejected based on studentized residuals procedure (if greater than 3). All statistical analyses were carried out using the Statistica v.11 (StatSoft Inc., USA).

3. Results

3.1. Ozone exposure, meteorological and soil conditions

Ozone was the most important air pollutant in this area with SO_2 and NO_x concentrations always within the range of monitor detection limits (1 nl l^{-1}; data not shown). Ambient O_3 concentrations increased from March to June (Fig. 1) reaching the hourly maximum of 68 nl l^{-1} in April (Table 3, Fig. 1). Ozone exposure indices and mean daily profiles for the different O_3 treatments are presented in Table 3 and Fig. 1 respectively. No significant differences among chambers or O_3 treatments were detected on prevailing meteorological conditions (Table 3).

Total soil mineral N background represented in N0 treatment was stable throughout the experiment averaging 10 mg N_{min} kg^{-1} (Fig. 2). Soil N content increased with each N dose, and values remained slightly higher than before the N addition. At the end of the experiment, N20 and N40 presented 18 mg N_{min} kg^{-1} and 27 mg N_{min} kg^{-1} respectively (means across O_3) compared to the background 10 mg N_{min} kg^{-1}.

3.2. Visible foliar injury

Different O_3-injury typology was observed depending on plant family: *Leguminosae* species showed brown-reddish necrotic spots on the upper surface of mature leaves; *Gramineae* species exhibited necrotic leaf tips and an obvious increase in foliar senescence; *Silene* showed similar symptoms to clover although very scarce. No O_3-induced visible injury was registered on plants exposed to ambient O_3 levels (NFA treatment), nevertheless O_3 significantly increased LDI_c by 31% and 48% in NFA+ and NFA++ treatments respectively compared with FA (mean across N treatments; Table 4). No significant differences were observed among N treatments, neither N modulated the foliar injury response to ozone (Table 4).

3.3. Biomass

The pasture life cycle experienced a slow growing period during the first 45 days (February–March, growth rate of 1.7 g dw day^{-1}; Fig. 3), then a very fast and short growing period during April until mid-May (46–85 DaE, mean growth rate across O_3 and N treatments of 8.5 g dw day^{-1}), and finally pasture growth rate declines until mid-June (86-116 DaE, average growth rate of 4.4 g dw day^{-1}), when plants were completely dry.

The first aboveground biomass harvest was sampled before starting the O_3 exposure, in order to test pasture growth homogeneity in all the experimental plots. At this time, seedlings showed a very low yield with a mean value across O_3 and N treatments of 76 g dw m^{-2}, representing 14% of the total pasture yield at the end of the growing cycle. No significant differences were found among chambers, although differences between OTCs and AA plots were significant at this early time (see Appendix).

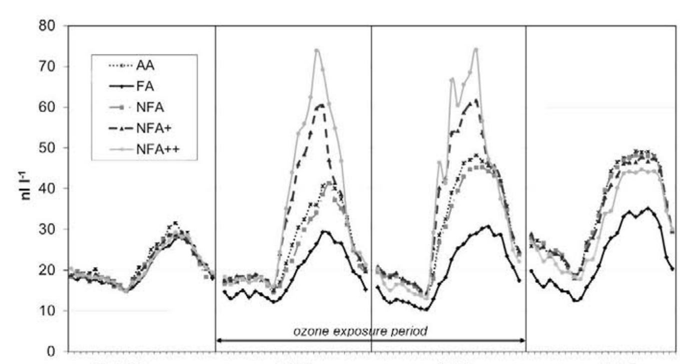

Fig. 1. Mean daily profiles of ozone concentration in the different treatments during the experimental period: AA = Ambient plots without chamber, FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3.

Table 3
Summary of the meteorological conditions and O_3 exposure indexes for the different O_3 treatments during the growing season (from February 25 to June 20) and during the maximum growth period (April–June). RH = relative air humidity; PAR = Photosynthetic Active Radiation; VPD = Atmospheric Vapor Pressure Deficit; FA = charcoal filtered air; NFA = non filtered air; NFA+ = non filtered air + 20 nl l^{-1} of O_3; NFA++ = non filtered air + 40 nl l^{-1} of O_3.

Meteorological conditions	February–June			April–June		
	24 h-mean	Max	Min	24 h-mean	Max	Min
Temperature (°C)	16.1	36.4	−1.5	18.8	36.4	6.1
RH (%)	65.6	99.6	17.5	61.1	99.2	17.5
PAR (μml m^{-2} s^{-1})	476.9	2248.2	0	550.7	2248.2	0
VPD (Kpa)	1.08	6.33	0	1.35	6.33	0.03
Soil temp. (°C)	17.2	31.6	4.2	20.1	31.6	13.2
Soil humidity (%)	21.7	40.1	18	20.1	27.1	18.0
Precipitation (mm)	148.1[a]			97.7		

Ozone indexes	24 h-mean (nl l^{-1})	Hourly maximum (nl l^{-1})	8 h-mean (nl l^{-1})	Febr.–June AOT40 (nl l^{-1} h)	April–June AOT40 (nl l^{-1} h)
AA	29	68	36	3780	3737
FA	20	48	23	174	158
NFA	28	65	34	2805	2798
NFA+	33	138	45	9205	9173
NFA++	34	189	50	14,257	14,245

[a] Total precipitation.

At the second aboveground biomass harvest (Table 4), the mean yield of the pasture was 406 g dw m^{-2} (overall mean across O_3 and N treatments); thus, the accumulated biomass from the emergence represented 75% of the total yield at the end of the life of the pasture. Ozone caused stronger effects than N in all the biomass parameters evaluated since the O_3 factor accounted for 43–55% (depending of the parameter) of the total variability of the data compared with the <3% assigned to the N factor, 16–17% to the interaction and 14% to the block effect caused by the soil heterogeneity of the field. Green, senescent and total aboveground biomass were negatively affected by O_3 exposure (Table 4). Significant reductions on green biomass of 7%, 14% and 25% were detected in NFA, NFA+ and NFA++ treatments respectively compared with FA (means across all N treatments), although the differences between FA and NFA treatments were not statistically significant. Ozone increased 40% the senescent biomass in both NFA+ and NFA++ compared to FA, resulting in a strong increment up to 90% in the NFA++ treatment of the senescent/green biomass ratio (Fig. 4). When total yield was considered, O_3 exposure induced 9%, 11% and 21% reductions in NFA, NFA+ and NFA++ respectively

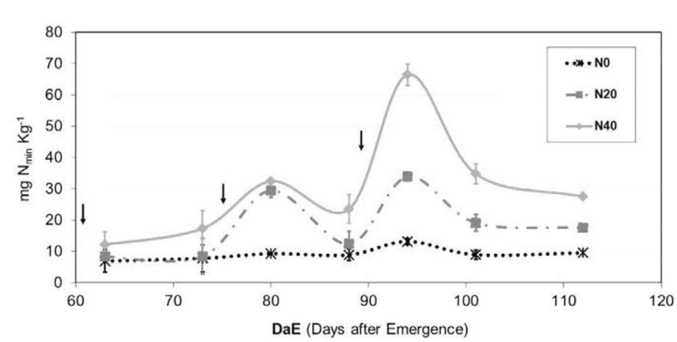

Fig. 2. Total soil N mineral content (N_{min}) for the different N treatments (means across O_3 treatments ± se). Arrows indicate the addition of the different N dose supplementation. N0 = soil background; N20 = 20 kg N ha^{-1}; N40 = 40 kg N ha^{-1}.

(mean across N treatments). No significant O_3 effects were detected on the fresh/dry weight ratio of green biomass (Table 4).

Nitrogen fertilization treatments did not cause any significant effect on biomass parameters in the second harvest. However, a significant interaction between O_3 and N was detected on green and total aboveground biomass (Table 4). While no differences among O_3 treatments were detected in the N0 treatment, NFA+ and NFA++ green biomass were significantly lower than FA in N20, and NFA++ biomass was significantly lower than FA and NFA in the N40 treatment (Fig. 5). Increasing N availability enhanced green biomass production mainly in FA and NFA, becoming higher than in the treatments supplemented with O_3. Thus, O_3 exposure reduced the fertilization effect of N additions. On the other hand, higher N availability in N40 decreased the O_3 induced effects on green biomass when O_3 concentrations were moderate, but not with high O_3 levels since green biomass in NFA++ was still significantly lower than in FA. Total biomass followed exactly the same statistical pattern as green biomass. The observed differences between OTCs and AA found in the earlier harvest disappeared in the second harvest (See Appendix).

A third aboveground biomass harvest was collected at the end of the life span of the pasture when all the species were completely dry. At this stage, a 19% yield reduction was observed in NFA++ compared with FA (Fig. 3), although this difference represented only a statistical trend ($p < 0.1$). No significant effect of the N treatments was observed on pasture yield at this harvest, neither a significant $O_3 \times N$ interaction.

Mean pasture root biomass per square meter collected at the second harvest was 37 g dw m^{-2} giving a mean root/aerial biomass rate of 0.1 (means across O_3 and N treatments). No significant effects caused by O_3, N or their interaction could be detected on root biomass or root/aboveground biomass ratio (Table 4).

3.4. Canopy gas exchange

NEE was affected by both O_3 and phenological stage factors without interactive effects between them (Table 5). No significant effects of the different N treatments were detected. NEE showed maximum values at the Reproductive Stage, when the pasture reached its maximum growth development. At this stage, mean values across N treatment averaged 26 μmol CO_2 m^{-2} s^{-1} (Fig. 6). During the Senescence Stage, NEE was reduced to 9 μmol CO_2 m^{-2} s^{-1}, thus 64% lower compared to the maximum (means across O_3 and N treatments). Ozone exposure induced a reduction of 28% of NEE throughout the growing cycle compared with FA (mean across N treatments and phenological stages), and O_3 ambient levels (NFA) were enough to produce this effect (Table 5). When NEE was expressed in terms of dry biomass (μmol CO_2 g dw^{-1} s^{-1}), the O_3 induced differences were significant only at the Vegetative Stage (Fig. 6b). Interestingly, maximum NEE values based on dry biomass were reached in the earlier stages of the pasture, diminishing progressively until pasture senescence (Fig. 6).

Dark respiration (R_{eco}) was relatively stable across O_3 and phenological stages (Table 5; Fig. 6) and no differences were detected caused by N treatments. However, when expressed in terms of dry weight biomass, both O_3 and phenological stage factors affected R_{eco} (Fig. 6): at the Vegetative Stage, R_{eco} in NFA++ treatment doubled the value of the FA control (mean across N treatments). This effect was also observed during the Senescence Stage. Average respiration of bare soil (CO_2 flux without plants inside the sampling ring) was −2.2 μmol CO_2 m^{-2} s^{-1}, representing about 28% of the average R_{eco} of the canopy pasture (−7.82 μmol CO_2 m^{-2} s^{-1}, average value across O_3 and N treatments). GPP presented the same phenological pattern as NEE, with maximum values recorded during the peak growing season

Table 4
Growth related parameters (mean ± se) corresponding to the second harvest for the different O_3 and N treatments (after 38 days of O_3 exposure). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air + 20 nl l^{-1} of O_3, NFA++ = non filtered air + 40 nl l^{-1} of O_3. Different letters indicate significant differences among treatments.

	Canopy leaf damage index (%)	Senescent biomass (g dw m^{-2})	Total aboveground biomass (g dw m^{-2})	Root biomass (g dw m^{-2})	Fresh/dry aerial biomass ratio	Senescent/green biomass ratio	Root/aerial biomass ratio
Experimental factors							
Ozone	<0.005	<0.05	<0.05	ns	ns	<0.05	ns
Nitrogen	ns	ns	ns	ns	ns	ns	ns
Nitrogen × Ozone	ns	ns	<0.05	ns	ns	ns	ns
Block	ns	ns	ns	ns	ns	ns	ns
Mean values							
FA N0	0	20.1 ± 3.1	417.9 ± 17.0^{abc}	37.7 ± 8.5	19.2 ± 2.0	0.05 ± 0.01	0.09 ± 0.02
NFA N0	0	20.8 ± 6.9	382.6 ± 14.6^{bc}	36.3 ± 5.6	20.9 ± 1.9	0.06 ± 0.02	0.10 ± 0.02
NFA+ N0	30.3 ± 3.9	48.5 ± 7.5	414.1 ± 11.1^{bc}	38.3 ± 7.4	17.7 ± 2.0	0.13 ± 0.02	0.10 ± 0.02
NFA++ N0	45.1 ± 2.7	42.5 ± 2.3	380.4 ± 10.6^{bc}	44.4 ± 14.9	21.1 ± 1.0	0.13 ± 0.01	0.13 ± 0.05
FA N20	0	32.9 ± 7.5	495.2 ± 20.3^{a}	39.1 ± 2.4	19.7 ± 0.4	0.07 ± 0.02	0.09 ± 0.01
NFA N20	0	16.1 ± 6.0	404.5 ± 23.5^{bc}	36.5 ± 3.1	20.2 ± 1.8	0.04 ± 0.01	0.09 ± 0.00
NFA+ N20	36.7 ± 3.4	35.7 ± 17.5	393.4 ± 46.6^{bc}	42.9 ± 8.2	20.4 ± 0.4	0.1 ± 0.04	0.12 ± 0.03
NFA++ N20	47.3 ± 4.1	36.6 ± 7.6	349.4 ± 39.5^{c}	23.6 ± 0.9	22.1 ± 1.1	0.12 ± 0.03	0.08 ± 0.01
FA N40	0	28.3 ± 10.5	442.0 ± 19.2^{ab}	47.0 ± 14.6	20.3 ± 3.0	0.07 ± 0.02	0.11 ± 0.04
NFA N40	0	17.5 ± 1.8	450.6 ± 18.9^{ab}	36.6 ± 0.6	21.3 ± 0.7	0.04 ± 0.01	0.08 ± 0.01
NFA+ N40	26.1 ± 2.6	30.4 ± 5.3	404.0 ± 26.0^{bc}	33.5 ± 4.7	20.0 ± 1.1	0.08 ± 0.02	0.09 ± 0.01
NFA++ N40	51.1 ± 3.9	35.4 ± 6.1	344.4 ± 25.1^{c}	28.9 ± 5.4	22.1 ± 1.5	0.11 ± 0.01	0.10 ± 0.02

(Reproductive Stage). Ozone reduced pasture GPP, being the NFA++ values 80% significantly lower than FA (Table 5). At the end of the growing cycle (Senescence Stage), due to the O_3-induced increment of R_{eco}, only FA sustained a positive gas exchange balance (Fig. 6).

4. Discussion

To our knowledge, the present experiment is the first one studying the response of an experimental annual pasture community to increased O_3 and N levels growing under natural soil conditions and allowing plant competition. Although growing inside OTCs, the pasture followed a similar development to pastures growing under natural conditions in the Central Iberian Peninsula. The average yield of the experimental pasture at maximum development was 406 g dw m^{-2}, equivalent to 4060 kg dry matter ha^{-1}. This value is within the range of 500–5400 kg dry matter ha^{-1} $year^{-1}$ reported under natural conditions (Olea and San-Miguel-Ayanz, 2006; Vázquez-De-Aldana et al., 2008; Hussain et al., 2009). Likewise, NEE and dark respiration rates were similar to values measured in natural annual pastures in Portugal (Hussain et al., 2009). Therefore, our experimental annual community can be considered representative of natural annual pastures.

Ozone clearly affected the annual pasture community causing visible foliar injury, accelerating plant senescence and reducing green and total aboveground biomass. Ozone induced foliar visible damage in 30%–48% of the canopy and decreased up to 25% of green biomass and up to 21% of total aboveground biomass. Interestingly, even ambient O_3 levels (NFA treatment) showed a clear trend to reduce total aboveground biomass of the annual community (9%). These results agree with previous studies performed with individual annual species or two-species mesocosms showing O_3-induced reductions on growth parameters within the range 20–30% in some sensitive species (Gimeno et al., 2004a,b; Sanz et al., 2005, 2007). Thus, the response to O_3 observed in an annual community was in the range of the effects reported for individual species. By contrast, well established perennial grassland communities have shown higher resilience to O_3 effects when comparing to individual component species (Volk et al., 2006; Stampfli and Fuhrer, 2010). On the other hand, our experimental annual community seemed to be more sensitive to O_3 that perennial grasslands since significant effects were detected after few weeks of O_3 exposure. Volk et al. (2011) did not find any O_3-response at community level after 5 years of O_3 exposure of a high

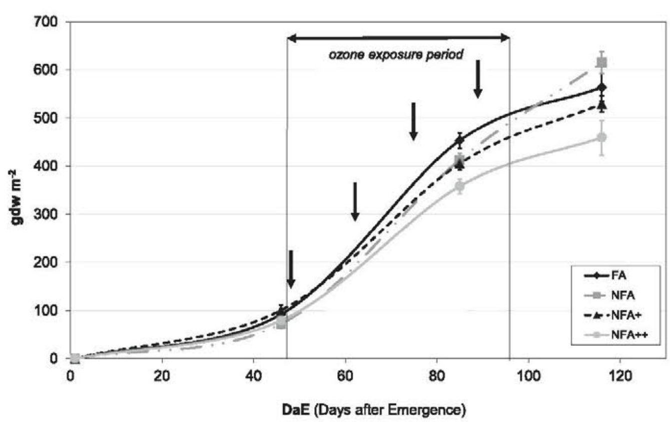

Fig. 3. Total aboveground biomass of the pasture for the different O_3 treatments since emergence till the end of the life span (means across N treatments ± se). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3. Arrows indicate the time of N addition.

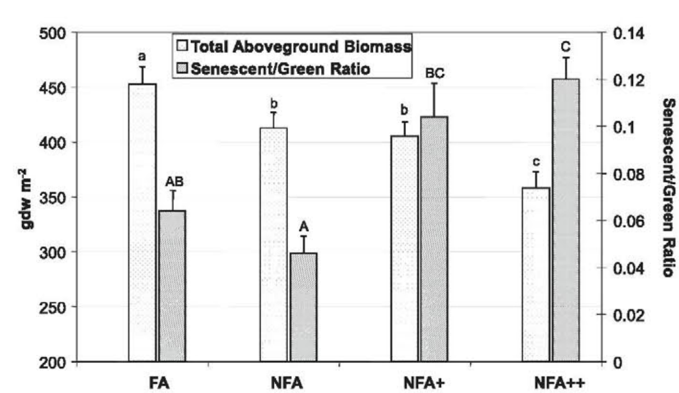

Fig. 4. Total aboveground biomass (g dw m^{-2}) and senescent/green biomass ratio for the different O_3 treatments at the second harvest (mean across N treatments ± se). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3. Different letters indicate significant differences among means.

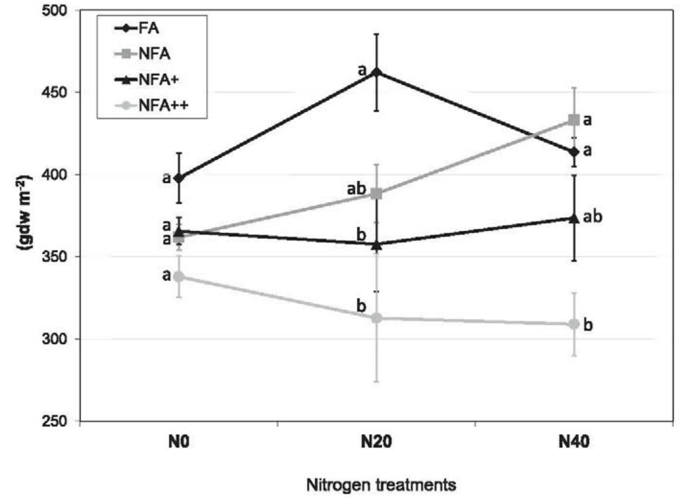

Fig. 5. Green biomass for the different O_3 and N treatments at the second harvest (mean across N treatments ± se). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3. N0 = soil N background, N20 = 20 kg N ha^{-1}; N40 = 40 kg N ha^{-1}. Different letters indicate significant differences among O_3 treatments.

diverse alpine perennial grassland. Similarly, only small O_3 effects on community composition were observed in calcareous grassland turfs after three growing seasons (Thwaites et al., 2006). The fact that annual communities regenerate each growing season from the seed bank with a fast growing rate when meteorological conditions are optimal, provides annual grasslands its characteristic state of non-maturity. In this sense, our results support the idea of Grime et al. (2000) that mature ecosystems exhibit greater inertia to stress and disturbance than newly established communities. Other sown model perennial communities have also shown quick and strong responses to O_3 suggesting that high growing rates is among

Table 5
Mean values of the canopy gas exchange parameters expressed in terms of ground surface area (μmol CO_2 m^{-2} s^{-1}) corresponding to the second harvest period for the different O_3 treatments and phenological stages (mean ± se). Values are averages across N treatments. FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3. NEE = Ecosystem CO_2 Exchange; R_{eco} = Ecosystem Dark Respiration; GPP = Gross Primary Production (GPP = NEE−R_{eco}). DaE = Days after Emergence. Different letters indicate significant differences among O_3 treatments (across N treatments and phenological stages) and differences among phenological stages (across N and O_3 treatments).

	NEE (μmol CO_2 m^{-2} s^{-1})	R_{eco} (μmol CO_2 m^{-2} s^{-1})	GPP (μmol CO_2 m^{-2} s^{-1})
Experimental factors			
Ozone	<0.05	ns	<0.01
Nitrogen	ns	ns	ns
Phenological stage	<0.001	ns	<0.001
O_3 × phenological stage	ns	<0.1	ns
Mean values per ozone treatment (across N treatments and phenological stages)			
FA	17.31 ± 1.60^{a}	-6.26 ± 0.56	8.77 ± 1.63^{a}
NFA	11.97 ± 1.66^{b}	-7.82 ± 0.65	3.78 ± 2.08^{ab}
NFA+	13.50 ± 1.10^{b}	-7.04 ± 0.52	6.22 ± 1.66^{ab}
NFA++	12.01 ± 2.10^{ab}	-10.19 ± 1.91	1.76 ± 3.14^{b}
Mean values per phenological stage (across N and O_3 treatments)			
Vegetative stage (47 DaE)	16.4 ± 1.14^{b}	-7.33 ± 0.84	9.08 ± 1.53^{a}
Reproductive stage (62 DaE)	25.81 ± 1.56^{c}	-8.05 ± 0.86	16.3 ± 2.67^{a}
Senescence stage (91 DaE)	9.20 ± 0.70^{a}	-7.82 ± 0.48	1.28 ± 1.12^{b}

the main plant trait related to O_3-sensitivity (see review in Bassin et al., 2007).

In addition to reductions in green and total aboveground biomass, O_3 significantly increased the senescent biomass of the annual pasture. The O_3-induction of premature senescence has been frequently observed in different vegetation types (Ashmore, 2005) and was also reported in previous experiments performed with annual single species (Bermejo et al., 2003; Sanz et al., 2007). Premature senescence together with reduction of aboveground biomass would imply lower plant resources for flower and seed development determining annual community viability. In fact, the O_3-induced reduction of the reproductive capacity of some annual legumes has been demonstrated in experiments with individual species (Gimeno et al., 2004b). Results of the O_3 effects on biomass, flower and seed production of the individual species forming this annual community are presented elsewhere (Calvete-Sogo et al., 2013).

The O_3 effects on pasture biomass were related to the observed response of gas exchange rates since reductions of NEE up to 28% were recorded. Similar results were observed when NEE was expressed based on dry biomass or on ground surface area. Thus O_3 effects on NEE at canopy level were partially explained by the O_3-induced reduction in biomass, but photosynthetic and/or respiration rates of the pasture were also affected. Indeed, O_3 increased dark respiration when calculated on biomass weight basis, but effects on photosynthetic rates cannot be disregarded (O_3 effects on photosynthesis at leaf level are presented elsewhere: Calvete-Sogo et al., 2013). Dark CO_2 fluxes include aboveground plant respiration, plant root respiration and soil microbial respiration, representing a combination of plant and soil processes. Since aboveground biomass was responsible of 70% of the observed dark respiration at the late phenological stage, effects on plant metabolism could mask the effects on soil processes. A test with a limited amount of replicated measurements showed that O_3 strongly increased bare soil dark respiration (−3.9 μmol CO_2 m^{-2} s^{-1} in NFA + compared to −1.78 μmol CO_2 m^{-2} s^{-1} in FA; data not shown) suggesting that O_3 could be affecting both plant and soil processes. Further research is needed to clarify possible O_3 effects on soil metabolism. The combined effects of reducing NEE and increasing dark respiration resulted in a significant O_3-induced reduction of GPP. Recent works indicate that O_3 can reduce C sequestration in tree living biomass (Harmens and Mills, 2012). However, quantifying the C budget of grassland systems is still challenging and further investigation is needed to quantify O_3 effects on C sequestration in annual grasslands.

Increasing N availability slightly affected annual pasture development. The low N doses used in this experiment, selected to reproduce the potential range of atmospheric N deposition in this area, increased soil mineral N content but these doses were not enough to significantly affect the yield or gas exchange rates of the annual pasture. The background soil N content of the soil (10 mg N_{min} kg^{-1} i.e. 33 kg N_{min} ha^{-1}) seemed to be enough to cover pasture N nutritional demand, explaining the lack of response to low N additions. Natural annual pastures usually grow in low fertility soils (Vázquez-De-Aldana et al., 2008) and a soil N content representing one tenth of the background soil N content of our experiment has been reported in natural annual pastures in Portugal (Hussain et al., 2009). Other nutrients, such as phosphorus, could be limiting plant development in this ecosystem type.

Despite the low responsiveness to N, a significant interactive response between N and O_3 was detected in green and total aboveground biomass production. On the one hand, O_3 reduced the fertilization effect of higher N availability since yield responses to N addition were positive only under low O_3 levels. This effect was not

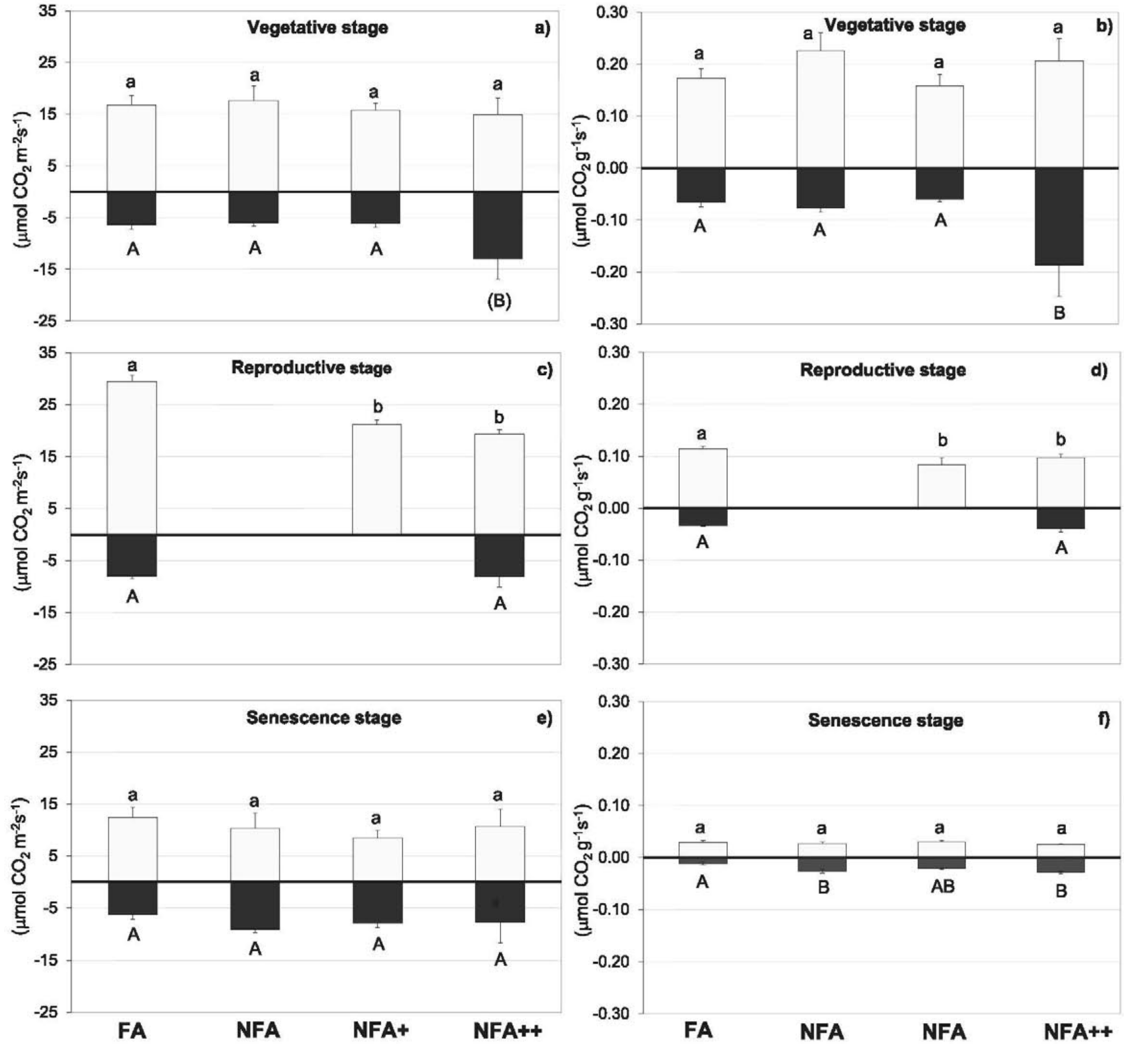

Fig. 6. Net Ecosystem gas exchange (NEE; gray bar) and Ecosystem Respiration (R_{eco}; dark bar) for the different O_3 treatments at three phenological stages. Values are expressed in terms of ground surface area (μmol CO_2 m^{-2} s^{-1}, left graphs) and dry weight biomass (μmol CO_2 g dw^{-1} s^{-1}, right graphs). FA = charcoal filtered air, NFA = non filtered air, NFA+ = non filtered air supplemented with 20 nl l^{-1} of O_3, NFA++ = non filtered air supplemented with 40 nl l^{-1} of O_3. Values are means across N treatments ± se. Different letters indicate significant differences among means for NEE and R_{eco}; letters within brackets indicate a trend ($p < 0.1$).

explained by a reduction of root growth or root/aboveground biomass ratio, suggesting that O_3 could be affecting plant metabolism related to nitrogen. On the other hand, N seemed to counterbalance O_3 yield reductions when pollutant levels were moderate, but this compensation was not observed in the high O_3 exposure treatment. A similar positive influence of N on O_3 effects on yield and quality parameters has been already detected when annual plants have been tested individually (Sanz et al., 2005, 2007, 2011). Other authors have highlighted the importance of nutrient availability in modifying O_3 responses of semi-natural vegetation (Bassin et al., 2007) but these interactions need further characterization in different vegetation types.

Ozone exposure-response functions were built based on the results obtained in this experiment using the relative yield loss of green and total aboveground biomass in the second harvest as the response parameter, and the accumulated O_3 exposure from the start of the O_3 exposure until the harvest (from 47 till 85 DaE, i.e. 38 days). Considering a 10% yield loss, the exposure-based critical level for annual communities would be 3545 nl l^{-1} h and 4249 nl l^{-1} h for green and total aboveground biomass respectively ($y = -0.0021x + 97.44$, $R^2 = 0.94$, $p < 0.05$ for green biomass and $y = -0.0016x + 96.80$, $R^2 = 0.84$, $p < 0.05$ for total aboveground biomass). These values are slightly above the critical level of 3000 nl l^{-1} h currently proposed for the protection of grasslands dominated by annuals species (CLRTAP, 2010). In any case, O_3 concentrations in the Iberian Peninsula where annual pastures grow frequently exceed these values (Fernández-Fernández et al., 2011) Also, critical levels are above the long-term objective of 3000 nl l^{-1} h established by the EU Air Quality Directive for plant protection, but below the current target of 9000 nl l^{-1} h defined by the Air Quality Directive to be met since 2010.

5. Conclusions

Ozone exposure induced visible injury and reduced the yield and gross primary production of a model annual community. Even ambient O_3 levels were enough to decrease aboveground biomass and NEE. Small increments of soil N availability slightly altered pasture growth but a significant interactive effect with O_3 exposure was detected. While O_3 limited the fertilization effect of higher soil

N availability, higher N could compensate O_3 effects on yield only when concentrations were moderate, but not under high O_3 levels. The response of the annual community was related to the cumulative O_3 exposure during the experiment suggesting an O_3 critical level of 4000 nl l^{-1} h to protect this vegetation type against a 10% yield loss. Since O_3 concentrations in the Mediterranean area frequently exceed that level, O_3 must be considered as a stress factor for annual pastures that can affect the yield, structure and composition of these ecosystems.

Acknowledgments

This research was funded by the Spanish projects MONTES (CONSOLIDER-INGENIO CSD2008-00040), AGRISOST (S2009AGR-1630, Comunidad de Madrid), EDEN (CGL2009-13188-C03-02) and the EU-FP7-ENV-2011 ECLAIRE project. The OTC field site at "La Higueruela Research Farm" was possible thanks to an agreement between CIEMAT and Museo de Ciencias Naturales/CSIC. Appreciative acknowledgment is extended to Carlos Lacasta Dutoit, José María Gómez Camacho and La Higueruela/CSIC research farm staff for their valuable suggestions in the experimental design and their technical support in the field work.

Appendix

Table A.1Edaphic and physico-chemical characteristics of Ap horizon.

Edaphic characteristics	Organic matter by oxidation (%)	1.26
	% Clay	11.4
	% Silt	24.0
	% Sand	64.6
Physic-chemical characteristics	pH	6.9
	Olsen P (ppm)	39
	Changes N (ppm)	62
	Changes K (ppm)	244
	Changes Ca (ppm)	1780
	Changes K (ppm)	160
N mineral Content	NO_3 (mg N/kg soil)	17.26
	NH_4^+ (mg N/kg soil)	0.16

Assessing the chamber effect: AA Plots vs. NFA OTCs

AOT40 values in NFA chambers were slightly lower (average difference of 25%) than in ambient air (Table 3). In regards to meteorological conditions, an increment of 1.6 of temperature and a decrease of 148 μmol m^{-2} s^{-1} in PAR were recorded inside the OTC compared to AA plots since plant emergence till the second harvest. The other meteorological parameters did not present important differences to be highlighted (differences in soil temperature <1 °C, in SWC <0.2 m^{-3} and in air relative humidity <4%). The growing pattern and canopy gas exchange values were compared between pasture growing in NFA chambers and in AA plots in order to control the chamber effect (Fig. A.1). Although no differences were recorded on species germination and distribution between AA and NFA plots, significant differences ($p < 0.05$) in total aboveground biomass were detected at the early stage of pasture development (Vegetative Stage, 46 DaE), when biomass inside the OTCs was almost 50% higher than in AA plots (mean value across N treatments; Fig. A.1). Similar results were found for green biomass. The same pattern was found in NEE values (Fig. A.1b), although these differences represented only a statistical trend ($p < 0.1$). This early delay in pasture development found in AA plots disappeared during the spring peak growth and no significant differences were recorded in the following biomass harvests or gas exchange assessments.

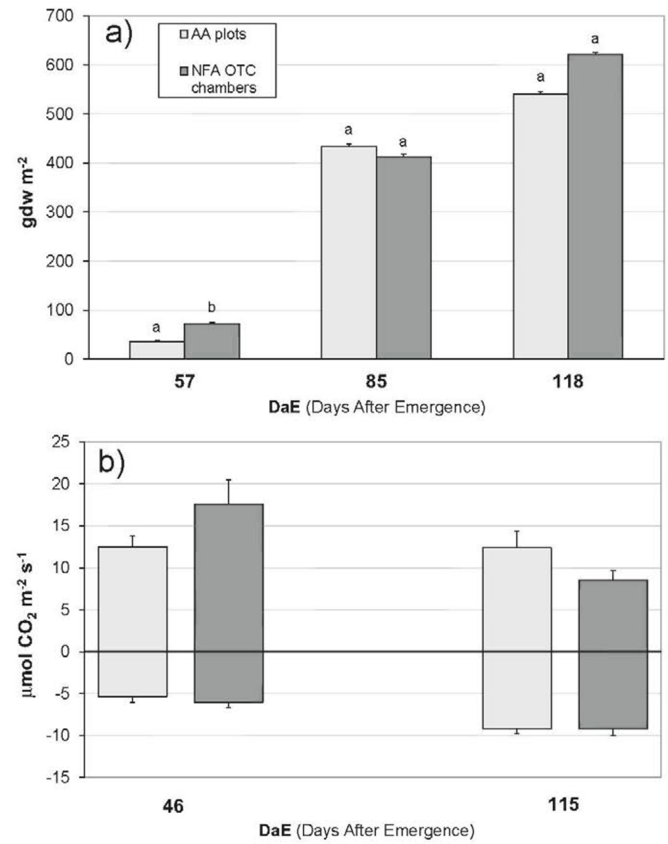

Fig A.1. Total aboveground biomass (a) and gas exchange (NEE and R_{eco}) (b) for ambient plots (AA) and non-filtered air chambers (NFA) to test the chamber effect. Different letters indicate significant differences between means (mean ± se).

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atmosenv.2014.05.073.

References

Ashmore, M.R., 2005. Assessing the future global impacts of ozone on vegetation. Plant Cell. Environ. 28, 949–964.

Àvila, A., Rodà, F., 2012. Changes in atmospheric deposition and streamwater chemistry over 25 years in undisturbed catchments in a Mediterranean mountain environment. Sci. Total Environ. 434, 18–27.

Bassin, S., Volk, M., Fuhrer, J., 2007. Factors affecting the ozone sensitivity of temperate European grasslands: an overview. Environ. Pollut. 146, 678–691.

Bermejo, V., Gimeno, B.S., Sanz, J., De la Torre, D., Gil, J.M., 2003. Assessment of the ozone sensitivity of 22 native plant species from Mediterranean annual pastures based on visible injury. Atmos. Environ. 37, 4667–4677.

Bermejo, V., Gonzalez-Fernandez, I., Calvo, E., Alonso, R., 2011. Ozone effects on crops in Mediterranean Europe. In: Mills, G., Harmens, H. (Eds.), Ozone Pollution: a Hidden Threat to Food Security. ICP Vegetation Programme Coordination Centre, UNECE-CLRTAP WGE, p. 112.

Bytnerowicz, A., 2002. Physiological/ecological interactions between ozone and nitrogen deposition in forest ecosystems. Phyt. Rei Bot. 42, 13–28.

Cape, J.N., 2008. Surface ozone concentrations and ecosystem health: past trends and a guide to future projections. Sci. Total Environ. 400, 257–269.

Calvete-Sogo, H., Bermejo, V., Sánchez, L., Muntifering, R., Sanz, J., Elvira, S., González-Fernández, I., Vallejo, A., Alonso, R., 2013. Ozone and nitrogen effects on the annual understory vegetation of open holm oak forest. IUFRO meeting (Vegetation Response Climate Change and Air Pollution – Unifying Evidence and Research across Northern and Southern Hemisphere), Ilhéus, Bahia, Brazil, September 1-6.

CLRTAP, 2010. Manual on Methodologies and Criteria for Modelling and Mapping Critical Loads and Levels, and Air Pollution Effects, Trends and Risks. Chapter 3: Mapping Critical Levels for Vegetation. Convention on Long-Range Transboundary Air Pollution. http://www.icpmappinmg.org/.

Cristofanelli, P., Bonasoni, P., 2009. Background ozone in the southern Europe and Mediterranean area: influence of the transport processes. Environ. Pollut. 157, 1399–1406.

Dupre, C., Stevens, C.J., Ranke, T., et al., 2010. Changes in species richness and composition in European acidic grasslands over the past 70 years: the contribution of cumulative atmospheric nitrogen deposition. Glob. Change Biol. 16, 344–357.

EEA, 2011. Air Quality in Europe – 2011 Report Technical Report No. 12/2011. European Environment Agency, Copenhagen.

Emmett, B.A., 2007. Nitrogen saturation of terrestrial ecosystems: some recent findings and their implications for our conceptual framework. Water Air Soil. Pollut. Focus 7, 99–109.

Fernández-Fernández, M.I., Gallego, M.C., García, J.A., Acero, F.J., 2011. A study of surface ozone variability over the Iberian Peninsula during the last fifty years. Atmos. Environ. 45, 1946–1959.

Ferretti, M., Bussotti, F., Calatayud, V., Schaub, M., Kräuchi, N., Petriccione, B., Sanchez-Peña, G., Sanz, M.J., Ulrichf, E., 2007. Ozone and forests in South-Western Europe. Environ. Pollut. 145, 617–619.

García, R.L., Norman, J.M., Mcdermitt, D.K., 1990. Measurement of canopy gas exchange using an open chamber system. Remote Sens. Rev. 5 (1), 141–162.

García-Gómez, H., Garrido, J.L., Vivanco, M.G., Lassaletta, L., Rabago, I., Àvila, A., Tsyro, S., Sánchez, G., González Ortiz, A., González-Fernández, I., Alonso, R., 2014. Nitrogen deposition in Spain: modeled patterns and threatened habitats within the Natura 2000 Network. Sci. Total Environ. 485–486, 450–460.

Gimeno, B.S., Bermejo, V., Sanz, J., De la Torre, D., Elvira, S., 2004a. Growth response to ozone of annual species from Mediterranean pastures. Environ. Pollut. 132, 297–306.

Gimeno, B.S., Bermejo, V., Sanz, J., De la Torre, D., Gil, J.M., 2004b. Assessment of the effects of ozone exposure and plant competition on the reproductive ability of three therophytic clover species from Iberian pastures. Atmos. Environ. 38, 2295–2303.

Grime, J.P., Brown, V.K., Thompson, K., et al., 2000. The response of two contrasting limestone grasslands to simulated climate change. Science 289, 762–765.

Harmens, H., Mills, G., 2012. Ozone Pollution: Impacts on Carbon Sequestration in Europe. Programme Coordination Centre for the ICP Vegetation. Centre for Ecology and Hydrology, Bangor, UK, ISBN 978-1-906698-31-7.

Hayes, F., Jones, M.L.M., Mills, G., Ashmore, M., 2007. Meta-analysis of the relative sensitivity of semi-natural vegetation species to ozone. Environ. Pollut. 146, 754–762.

Heck, W.W., Taylor, O.C., Adams, R., Bingham, G., Miller, H., Preston, E., Weinstein, L., 1982. Assessment of crop loss from ozone. J. Air Pollut. Control Assoc. 32, 353–361.

Hussain, Z., Kemp, P.D., Horne, D.J., Jaya, I.K.D., 2009. Pasture production under densely planted young willow and poplar in a silvopastoral system. Agrofor. Syst. 76, 351–362.

Millán, M.M., Mantilla, E., Salvador, R., et al., 2000. Ozone cycles in the western Mediterranean basin: interpretation of monitoring data in complex coastal terrain. J. Appl. Meteorol. 39, 487–508.

Montoya, J.M., Meson, M., Ruiz, J., 1988. Una dehesa testigo, La dehesa de Moncalvillo, Serie Técnica num 5. Ministerio de Agricultura Pesca y Alimentación, ICONA, p. 134.

Ochoa-Hueso, R., Allen, E.B., Branquinho, C., et al., 2011. Nitrogen deposition effects on Mediterranean-type ecosystems: an ecological assessment. Environ. Pollut. 159, 2265–2279.

Olea, L., San-Miguel-Ayanz, A., 2006. The Spanish dehesa. A traditional Mediterranean silvopastoral system linking production and nature conservation. In: Lloveras, J., González-Rodríguez, A., Vázquez-Yañez, O., Piñeiro, J., Santamaría, O., Olea, L., Poblaciones, M.J. (Eds.), Sustainable Grassland Productivity: Proceedings of the 21st General Meeting of the European Grassland Federation, pp. 1–15.

Sanz, J., Muntifering, R.B., Bermejo, V., Gimeno, Bs, Elvira, S., 2005. Ozone and increased nitrogen supply effects on the yield and nutritive quality of *Trifolium subterraneum*. Atmos. Environ. 39, 5899–5907.

Sanz, J., Bermejo, V., Gimeno, B.S., Elvira, S., Alonso, R., 2007. Ozone sensitivity of the Mediterranean terophyte *Trifolium striatum* is modulated by soil nitrogen content. Atmos. Environ. 41, 8952–8962.

Sanz, J., Bermejo, V., Muntifering, R.B., González-Fernández, I., Gimeno, B.S., Elvira, S., Alonso, R., 2011. Plant phenology, growth and nutritive quality of Briza maxima: responses induced by enhanced ozone atmospheric levels and nitrogen enrichment. Environ. Pollut. 159, 423–430.

Stampfli, A., Fuhrer, J., 2010. Spatial heterogeneity confounded ozone-exposure experiment in semi-natural grassland. Oecologia 162, 515–522.

Stevens, C.J., Dise, N.B., Mountford, J.O., Gowing, D.J., 2004. Impact of nitrogen deposition on the species richness of grasslands. Science 303, 1876–1879.

Sutton, M.A., Howard, C.M., Erisman, J.W., Billen, G., Bleeker, A., Grennfelt, P., Grinsven, H.V., Grizzetti, B. (Eds.), 2011. The European Nitrogen Assessment. Cambridge Univ. Press.. Available at: http://go.nature.com/5n9lsq.

Thwaites, R.H., Ashmore, M.R., Morton, A.J., Pakeman, R.J., 2006. The effects of tropospheric ozone on the species dynamics of calcareous grassland. Environ. Pollut. 144, 500–509.

Vázquez-De-Aldana, B.R., García-Ciudad, A., García-Criado, B., 2008. Interannual variations of above-ground biomass and nutritional quality of Mediterranean grasslands in Western Spain over a 20-year period. Aust. J. Agric. Res. 59, 769–779.

Volk, M., Bungener, P., Contat, F., Montani, M., Fuhrer, J., 2006. Grassland yield declined by a quarter in 5 years of free-air ozone fumigation. Glob. Change Biol. 12, 74–83.

Volk, M., Obrist, D., Novak, K., Giger, R., Bassin, S., Fuhrer, J., 2011. Subalpine grassland carbon dioxide fluxes indicate substantial carbon losses under increased nitrogen deposition, but not at elevated ozone concentration. Glob. Change Biol. 17, 366–376.

Wyness, K., Mills, G., Jones, L., Barnes, Jd, Jones, D.L., 2011. Enhanced nitrogen deposition exacerbates the negative effect of increasing background ozone in *Dactylis glomerata*, but not *Ranunculus acris*. Environ. Pollut. 159, 2493–2499.